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Perspective

Multiplex Biosensing for Simultaneous Detection of Mutations in SARS-CoV-2

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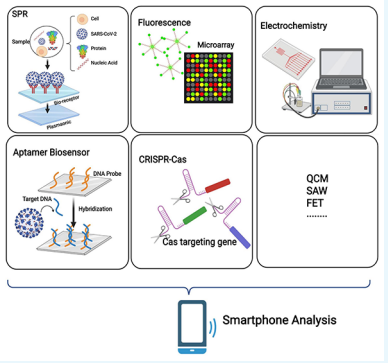
ABSTRACT: COVID-19 pandemic caused by the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) has become the world's largest public health emergency of the past few decades. Thousands of mutations were identified in the SARS-CoV-2 genome. Some mutants are more infectious and may replace the original strains. Recently, B.1.1.7(Alpha), B1.351(Beta), and B.1.617.2(Delta) strains, which appear to have increased transmissibility, were detected. These strains accounting for the high proportion of newly diagnosed cases spread rapidly over the world. Particularly, the Delta variant has been reported to account for a vast majority of the infections in several countries over the last few weeks. The application of biosensors in the detection of SARS-CoV-2 is important for the control of the COVID-19 pandemic. Due to high demand for SARS-CoV-2 genotyping, it is urgent to develop reliable and efficient systems based on integrated multiple biosensor technology for rapid detection of multiple SARS-CoV-2 mutations simultaneously. This is important not only for the detection and analysis of the current but also for future mutations. Novel biosensors combined with other technologies can be used for the reliable and effective detection of SARS-CoV-2 mutants.



MUTATIONS IN SARS-COV-2

Mutations are common and play an important role in the life cycle of viruses. For example, the *A82V* mutation in the glycoprotein of Ebola virus increases its infectivity.[1] A tyrosine-to-alanine mutation at residue 76 (*Y76A*) in the cytoplasmic tail region of M2 protein of influenza A is essential for the virus production.[2]

The worldwide spread and replication of SARS-CoV-2 provides a good opportunity for its mutation. Generally, the presence of RNA proofreading enzyme (NSP14)[3] in the genome of SARS-CoV-2 leads to fewer gene mutations in SARS-CoV-2 than in other RNA viruses. However, sequencing of several thousand SARS-CoV-2 genomes revealed a number of repeated mutations, including synonymous and non-synonymous (Figure 1A). Globally, NSP1/NSP2/NSP3/NSP11 are the most frequently mutated nonstructural genes and ORF7a/ORF3a/S are the most frequently mutated structural genes.[4] Most mutations in SARS-CoV-2 genome are silent and do not cause structural changes in encoded proteins. However, changes in SARS-CoV-2 surface proteins usually have more significant impact. SARS-CoV-2 genome encodes four structural proteins: Spike (S) protein, Envelope (E) protein, Membrane (M) protein, and nucleocapsid (N) protein. S protein is composed of S1 and S2 subunits responsible for binding to the receptor and for fusion with the cell membrane (Figure 1A).[5] SARS-CoV-2 enters host cells and replicates by fusing its spike glycoprotein with ACE2 on the surface of host cells.[6] Most SARS-CoV-2 vaccines and antibodies target S protein. Because SARS-CoV-2 S protein is important for recognizing the host cell surface receptor and mediating cell fusion, investigation of S protein mutations is of the utmost importance. The increasing frequency of spike amino acid variants in many geographic regions was identified by monitoring GISAID data.[7] According to WHO new rule on the name of variants, various strains can be recorded as B.1.1.7(Alpha), B1.351(Beta), P.1(Gamma), B.1.617.2(Delta), B.1.617.1(Kappa), P.2(Zeta), etc.[8]

D614G MUTATION

SARS-CoV-2 D614G mutant has attracted global attention. In the past year, this mutant has replaced the original SARS-CoV-2 and has become dominant in the pandemic. *D614G* is a missense mutation caused by A-G nucleotide mutation at position 23403 and the resulting amino acid change from aspartic acid (D) to glycine (G) at the position 614 of the S protein.[9] *D614G* mutation may lead to destabilization of the interaction between S1 and S2 domains.[10] *D614G* mutation does not enhance the affinity between ACE2 and S protein but

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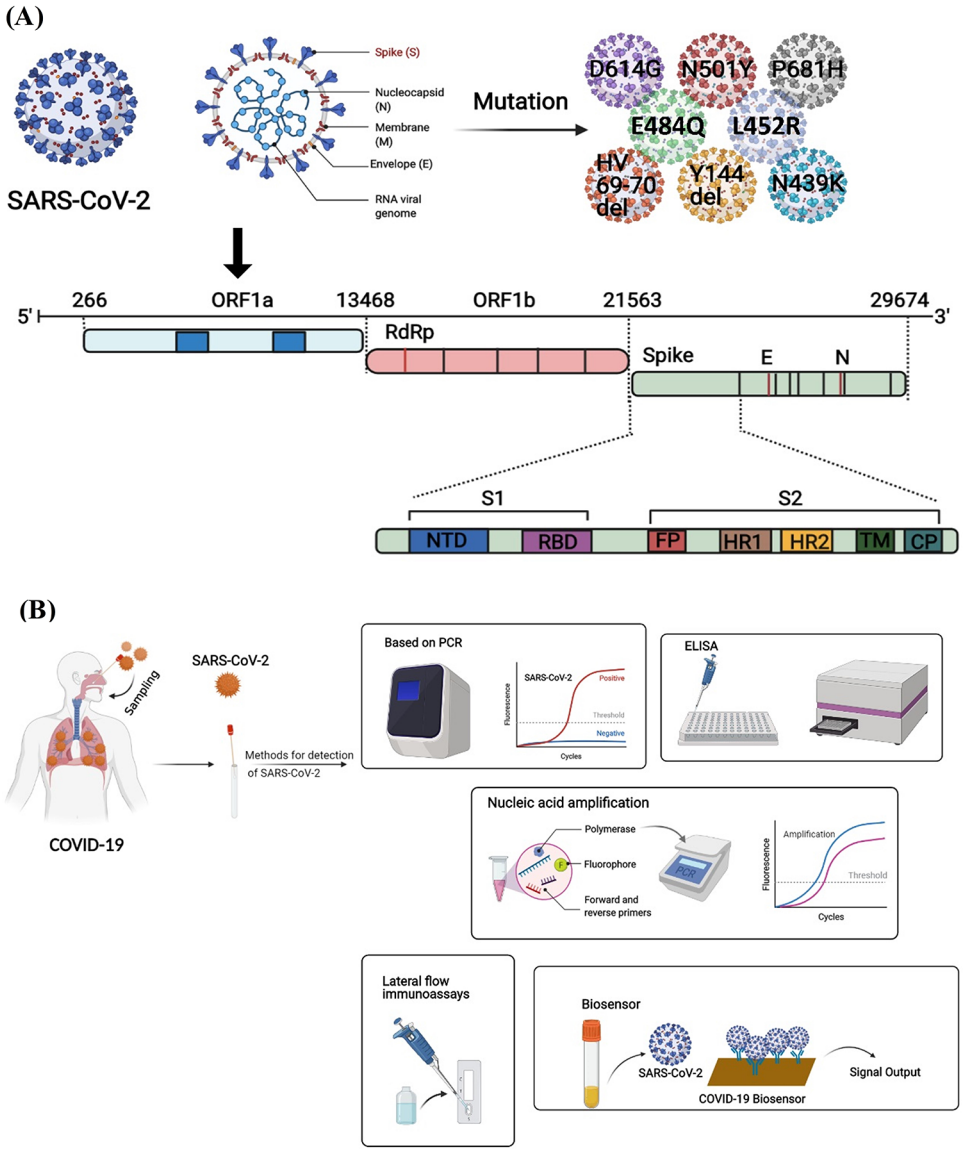

Figure 1. Mutations in SARS-CoV-2 and the methods of detecting SARS-CoV-2. (A) Structure of SARS-CoV-2 and the most common mutations (*D614G*, *N501Y*, *P618H*, *HV69-70del*, *Y144del*, and *N439K*) occurring in the process of transmission. (B) Methods for the SARS-CoV-2 detection include those based on ELISA, nucleic acid amplification, lateral flow immunoassays, and biosensors (figure was created in part with BioRender).

increases the amount of fully functional S protein on the virus surface. This in turn increases its chance to bind to host cells and improves the infection efficiency.[11] S^{G614} is more stable than S^{D614}, thus increasing the transmission efficiency of the S^{G614} mutant. Pseudovirus detection showed that ACE2 was the receptor of both *D614* and *G614*, but there was a functional difference.[12] *D614G* mutation enhanced the cleavage of S protein by protease and significantly promoted the entry of the virus into ACE2 expressing cells. The conformation of the *D614G* mutant makes the virus membrane more likely to fuse with the target cell membrane. This can change the binding characteristics of ACE2 through the allosteric effect, improve the flexibility of receptor-binding domain (RBD), and make the structure of the S protein more open and easier to bind with ACE2. *D614G* mutants showed increased viral load and transmission ability due to their higher replication capacity.[13] The higher titer of pseudovirus *in vitro* and the higher level of virus RNA in *G614* infected samples showed that *G614* was more infectious.[14] However, the *D614G* mutation did not show more resistance to neutralizing antibodies.[15] Furthermore, no clinical differences in the severity of symptoms were identified and the *D614G* mutation did not seem to affect the effectiveness of the vaccines targeting S protein.[10]

Notably, the latest experimental results discussed below suggest that SARS-CoV-2 infectivity is mainly determined by RBD mutations. The main SARS-CoV-2 variations circulating in the population, such as Alpha, Beta, Gamma, Delta, and others are also listed by their RBD mutations. At this time, there is no definitive support for the roles of the variations at other places, such as D614G, for SARS-CoV-2 infectivity.

MUTATIONS IN B.1.1.7(ALPHA) LINEAGE

Recently, a more transmissible strain, B.1.1.7(Alpha) (VUI-202012/01), has emerged, which has been spreading rapidly. B.1.1.7(Alpha) mutation caused a large number of infections in London and Kent in early December.[16,17] In addition,

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B.1.1.7(Alpha) has been found in many other countries and regions.

A number of variations have been identified in the new strain of B.1.1.7(Alpha), including 6 synonymous mutations (nonamino acid substitution), 3 frame deletions, and 14 nonsynonymous mutations (amino acid substitution). Five of the six synonymous mutations were in ORF1ab (C913T, C5986T, C14676T, C15279T, C16176T) and one in the M gene (T26801C).[18] Among these 17 mutations, multiple mutations were discovered in the spike gene (*deletion 69-70, deletion 144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H*).[18] Interesting SARS-CoV-2 features include binding of the amino acids of six key interfaces on RBD to ACE2, and the furin protease cleavage site of SARS-CoV-2. *N501Y* mutation occurred in the RBD of S protein at position 501, where asparagine (N) was replaced by tyrosine (Y).[19] *N501Y* mutation changes the virus envelope surface protein and increases its ability to enter human cells and to bind to the ACE2 receptor. *P681H* mutation occurred in S1/S2 adjacent to the furin cleavage site. Furin cleavage decreased the stability of S protein, thus exposing the open domain. As a result, the binding affinity between the S protein and ACE2 receptor was greatly improved, and the binding ability and infectivity to the virus were enhanced.[20] The deletion of two amino acids (H69 and V70) at positions 69 and 70 in the spike is one of many repeated deletions observed in the N-terminal domain of S protein. This double deletion may lead to conformational changes of S protein.[21] Transmissibility of B.1.1.7(Alpha) mutation appears to be significantly enhanced.

At present, there is no evidence that new strains lead to higher mortality or affect the efficacy of the vaccine.

OTHER HOT SPOT MUTATIONS OF SARS-COV-2

Although *N501Y* mutation and several other mutations also appeared in strain, called *501Y.V2* (B1.351(Beta)), it was completely independent of the B.1.1.7(Alpha). The *501Y.V2* mutant strain has stronger infectivity. The transmission rate is increased by 50%, and the virulence of this strain also appears to be increased.[21] Most of the new cases in Africa are caused by this mutant strain, but as the detection capacity of novel coronavirus (NCV) in Africa lags behind other regions, the prevalence of NCV circulating there is unclear. 501Y.V2 may also become a new dominant strain. Several mutations in the S protein, including *K417T, E484K*, and *N501Y*, and no deletion at 69/70 were detected in this SARS-CoV-2 variant. *E484K* may cause spatial interference in antibody recognition because negatively charged glutamate is replaced by positively charged lysine.

A third novel variant strain has emerged in Nigeria, which contains a *P681H* mutation in B.1.1.7(Alpha) pedigree. There are some similarities between this strain and the previous two strains, but these similarities may be caused by independent evolution. One of the prolines (P) in S protein was replaced by histidine (H) in *P681H* mutation. The substitution site is located at the site where "TMPRSS2 enzyme" cleaves S protein; therefore, this mutation can lead to increased infection ability.[22] *N439K* is a common receptor-binding motif (RBM) mutation. SARS-CoV-2 RBM, which is the main target of RBD neutralizing Ab reaction, is a highly variable S protein region in circulating virus. The N439K variant has C to A modification at the third codon position. *N439K* S protein enhanced the binding affinity between the hACE2 receptor and the *N439K* virus. Compared to wild type, *N439K* S protein had similar in vitro replication fitness and a similar clinical profile.[23]

"Double mutation" virus has also affected many countries. The official name of "double mutation" virus is new coronavirus variant B.1.617.2(Delta). According to the genomic data, B.1.617.2(Delta) may be more infectious than the wild-type coronavirus. Its transmission speed is similar to that of B.1.1.7(Alpha), but higher than that of B.1.351. Main B.1.617.2(Delta) mutations are *E484Q* and *L452R*. Although several other mutations were detected in this variant, they do not appear to have a significant effect on the characteristics and behavior of the virus. *E484Q* refers to the substitution of glutamic acid (E) at site 484 of S protein by glutamine (Q). The corresponding nucleotide change is the substitution of guanine (G) at site 23012 by cytosine (C) (G23012C). Compared to other variants, *E484Q* endows B.1.617.2(Delta) with stronger binding potential to hACE2 and better ability to escape the host immune system. *L452R* refers to the substitution of leucine (L) by arginine (R) at the 452 site of S protein. The corresponding nucleotide change is the substitution of thymine (T) at site 22917 by guanine (G) (*T22917G*). *L452R* mutation leads to a stronger affinity of S protein for the receptor, reduction of the immune system recognition ability, and increased resistance to antibodies. Vaccine breakthrough infections are of the major concern at this point. *E484Q* and *L452R* mutations may increase the ability of the virus to attach to cells and spread, thus increasing virus infectivity and affecting vaccine effectiveness. Although B.1.617.2(Delta) might have increased ability to escape the immune system, the existing vaccines may still have a protective effect; however, this will require further investigation.

The microevolution leads to constant changes of SARS-CoV-2. SARS-CoV-2 mutants were also found in animals. SARS-CoV-2 variant (called P.1) was identified, which is a branch of B.1.1.28 lineage.[24] This lineage contains 17 unique amino acid changes and 3 deletions. This variant contains three mutations in the spike receptor-binding domain: *K417T, E484K*, and *N501Y*.[25] van Dorp et al.[26] screened the SARS-CoV-2 genome isolated from minks to determine the existence of common repeated mutations in minks. Several new SARS-CoV-2 mutants were found in patients with low immunity, thus highlighting the rapid evolution of SARS-CoV-2.[27] Efficient and reliable detection of multiple mutations of SARS-CoV-2 is therefore crucial for the prevention of the spread of SARS-CoV-2 variants and control of pandemic.[28]

CURRENT METHODS FOR THE DETECTION OF SARS-COV-2 AND ITS MUTANTS

Virus infections are among the main causes of human morbidity and mortality. A number of techniques and methods have been developed to detect viruses, bacteria, and other pathogens, including colorimetry, fluorescence polarization, and electrochemical analysis.[29] Diagnosis of viral infections includes direct detection and serological methods. Viruses can be isolated from cell culture to identify their nucleic acids or antigens.[30] Nuclear acid sequence-based amplification (NASBA), restriction enzyme analysis (REA), direct immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), and reverse transcription polymerase chain reaction (RT-PCR) are among the most frequently used methods for virus detection.[31] Multiple PCR can simultaneously amplify several nucleic acid targets in a single sample. The standard

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Table 1. Current Methods for Detection of SARS-CoV-2

detection method	detection time	advantage	disadvantage	reference
RT-PCR	4−6 h	high credibility, high sensitivity	slow, equipment requirement	43, 44
ELISA	30 min to 2 h	simple, good specificity	unstable, low sensitivity, slow, false negative	37, 45
lateral flow immunoassays (LFIA)	15 min	cheap, fast, no sample pretreatment, multianalyte testing	low sensitivity, false-negative	46
CRISPR	40 min	fast, high sensitivity, multiplexing capability	off-target phenomenon	47

Table 2. Application of Biosensor Technology in Virus Detection[a]

virus	biosensor technology	recognition element	LOD	reference
HAV	electrochemistry	ssDNA	6.94 ng/L	107
HBV	electrochemistry	ssDNA (gold nanorods)	2.0×10^{-12} mol/L	108
HBV	LSPR	Antibody	100 pg/L	110
HBV	SERS	DNA	1.4×10^{-16} mol/L	109
HBV	SiNW/FET	ssDNA	3.2×10^{-15} mol/L	111
HCV	SWNT/FET	peptide nucleic acid (PAN)	5.0×10^{-13} mol/L	112
HIV	electrochemistry	ssDNA	7.05×10^{-12} mol/L	113
HIV	SiNW/FET	Antibody	4.0 mg/L	114
zika virus	electrochemistry	ssDNA	2.5×10^{-8} mol/L	115
zika virus	G/FET	Antibody	4.5×10^{-10} mol/L	116
dengue virus	electrochemistry	DNA	2.0×10^{-15} mol/L	117
dengue virus	LSPR	Antibody	0.06 mg/L	118
ebola virus	electrochemistry	DNA	4.7×10^{-9} mol/L	119
ebola virus	G/FET	Antibody	2.4 ng/L	120
influenza A virus	electrochemistry	hemagglutination	0.015 HAU pre probe	121
H3N2	SER	aptamer	10^{-4} HAU pre probe	122
H7N9	SPR	antibody	144 copies/mL	123
influenza A virus	CNT/FET	DNA	1.0×10^{-12} mol/L	124
MERS-CoV	electrochemistry	antibody	400 pg/L	125
SARS-CoV	LSPCF	antibody	0.1 ng/L	126
SARS-CoV-2	SPR(gold nanorods)	antibody	111.11 deg/RIU	127
SARS-CoV-2	LSPR	DNA	2.2×10^{-13} mol/L	59
SARS-CoV-2	G/FET	antibody	16 pfu/mL	56

[a]HAU: hemagglutination; LSPCF: localized surface plasmon coupled fluorescence; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

method for the detection of PCR products, agarose gel electrophoresis, is usually combined with Southern blot to determine sequence specificity. However, this method is complex and time consuming.[32]

Early detection can prevent SARS-CoV-2 from spreading and help control the pandemic. SARS-CoV-2 can be identified by nucleic acid detection and serological detection.[33,34] The virus in the lysate of host cells was detected by amplification and sequencing. Reverse transcription PCR (RT-PCR) is the gold standard for the detection of SARS-CoV-2.[35] However, SARS-CoV-2 is an RNA virus. If not stored properly or submitted for examination in time, it can degrade, leading to false-negative results.[36] Moreover, the immunoassay based on antigen−antibody interaction can also be used for the detection of SARS-CoV-2.[37]

Genomic sequencing and phylogenetic analysis are often used to identify mutations in SARS-CoV-2, which is helpful to understand the roles of point mutations in the transmissibility and toxicity of SARS-CoV-2.[10,38,39] For example, a combination of antibody detection and RT-PCR has been used to detect D614G mutant.[40]

However, these traditional methods are often complex, cumbersome, and time consuming. Furthermore, they require specific equipment and professionals to operate (Table 1). To overcome these shortcomings, biosensors can be used to detect SARS-CoV-2 and its mutations. With the increasing number of virus variants, it is urgent to develop efficient, rapid, and multiple biosensor technologies for simultaneous detection of SARS-CoV-2 mutations (Figure 1B).[41,42]

The genetic evolution of SARS-CoV-2 RBD may be affected by host gene editing, virus proofreading, random genetic drift, and natural selection, thus resulting in the emergence of more infectious variants.[48] SARS-CoV-2 mutations can lead to the reduction of the neutralization of antibodies. S protein mutation may weaken the binding between SARS-CoV-2 S protein and antibodies, thus potentially reducing the efficiency and efficacy of the existing vaccines and of the antibody therapy and increasing the SARS-CoV-2 transmission and infectivity. The change of binding free energy (BFE) between RBD and ACE2 can predict the impact of mutations on SARS-CoV-2 infectivity based on artificial intelligence prediction and genotyping of tens of thousands of patient genomic isolates. Delta variant contains both T478K and L452R. It is the most infectious strain among all variants with infectivity about 4 times that of the original virus. Detailed studies on how each RBD mutation affects infectivity and antibodies can be found on the mutation analyzer (Table 2).[48,49]

The mutation of COVID-19 diagnostic target may lead to the destruction of the diagnostic reagent currently used, resulting in false-positive and false-negative detection. The

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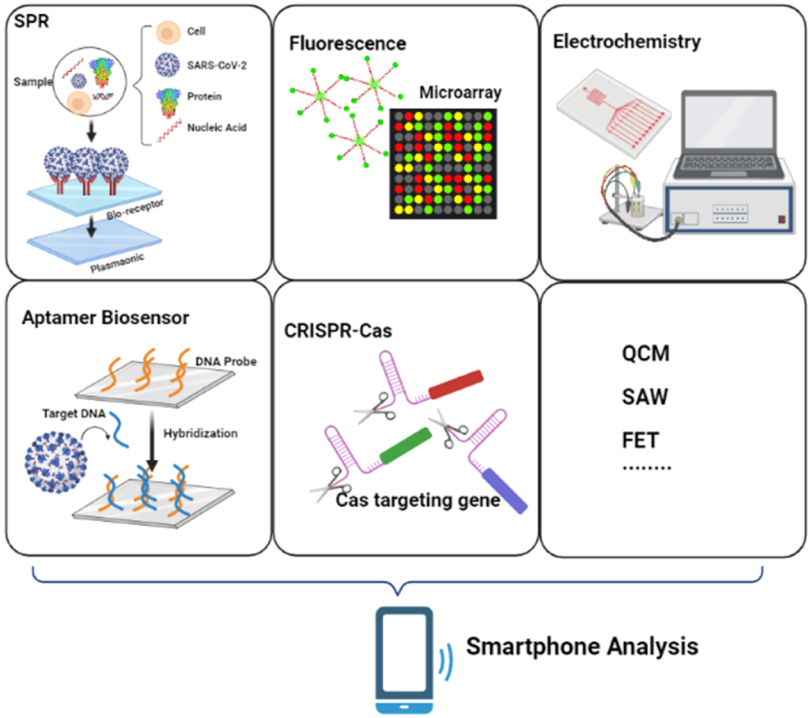

Figure 2. Biosensor technology for detection of SARS-CoV-2. The specific signals generated by biosensors can be used to detect SARS-CoV-2 and its mutation using a variety of detection principles, such as SPR, fluorescence, electrochemistry, aptamer based, CRISPR-Cas, and others. Digital intelligence can be applied for the point-of-care testing (figure was created in part with BioRender).

location, frequency, and coding protein of SARS-CoV-2 mutation were identified and analyzed in the global environment recently.[50] To avoid mutation prone diagnostic regions, diagnostic target selection and probe optimization should be based on nucleotide and gene mutation frequency analysis.[50]

BIOSENSOR TECHNOLOGY FOR THE DETECTION OF SARS-COV-2

Standard Biosensors. Biosensors have been used to detect different types of viruses, including human coronavirus,[51] HIV,[52] hepatitis virus,[53] Zika virus,[54] and various influenza viruses.[55] Biosensor technology can also be applied for efficient and reliable detection of SARS-CoV-2 and its variants. Seo et al. developed a field-effect transistor (FET) biosensor by coating graphene sheet with an antibody against SARS-CoV-2 S protein for SARS-CoV-2 detection in clinical samples.[56] Yadav et al. proposed a novel dielectric modulated field-effect transistor (FET) biosensor, which was improved by combining with a bimetallic control gate. The biosensor detects the spike and envelope proteins of SARS-CoV-2 by direct current and radio frequency parameters of charge density in viral proteins.[57] Murugan et al. designed two field-deployable portable plasma optical fiber absorbance biosensor (P-FAB) devices to detect N protein of virus directly from saliva.[58] Qiu et al. developed a bifunctional plasma biosensor combining plasma photothermal (PPT) effect and local surface plasmon resonance (LSPR) sensing transduction. The bifunctional plasma biosensor containing a Au nanoabsorbent (AuNIs) chip selectively detects specific sequences from SARS-CoV-2 by nucleic acid hybridization.[59] Arshak et al. reported detection of SARS-CoV-2 by field-effect sensors (FEDs). FEDs can directly identify the complete virus, the virus antigen, the virus nucleic acid, or the antibody produced by the immune system through the complete particle charge.[60]

Using antibodies, enzymes, or aptamers, novel biosensors can be designed to detect coronaviruses. Peng et al. developed a novel near-infrared phase-modulated plasma biosensor for the sensitive detection of SARS-CoV-2 and its spike glycoprotein.[61] Chen et al. reported modification of aptamers and their binding to SARS-CoV-2 N protein. The diluted SARS-CoV-2 N protein in human serum was detected by aptamer, and enzyme-linked aptamer binding assay (ELAA) was performed. Biotinylated ssDNA was used to detect N protein and then the horseradish peroxidase (HRP) conjugation system was used to detect N protein.[62] The formation of G-quadruplex interacts with the viral helicase NSP13, which can catalyze the unfolding of G-quadruplex and promote the transcription and replication of SARS-CoV-2. The g-tetraploid structure was found in the SARS-CoV-2 genome, which can be used as a target for antiviral drugs.[63] The G-quadruplex-based biosensor can be used as a potential tool to detect SARS-CoV-2.[64] Monoclonal antibodies against ACE2 and S1 were used as capture and detection probes in lateral flow immunoassays. The SARS-CoV-2 S1 protein was selectively detected by the lateral flow immunoassay based on ACE2, in which the binding of protein with membrane-bound antibody led to the change of cell bioelectricity.[65] Kim et al. developed a SARS-CoV-2 specific biosensor based on LFIA and used scFv-Fc antibody to distinguish different coronaviruses.[66] The detection of SARS-CoV-2 by biosensors based on electrical and optical signals, such as surface-enhanced Raman scattering (SERS), has also been reported.[67]

A biosensor might also be a good tool for the detection of SARS-CoV-2 mutation. The surface plasmon resonance (SPR) technique was used to study the effect of *D614G* on the binding kinetics of SARS-CoV-2 S protein with human ACE2.[68] SPR-based biosensors can be constructed to distinguish *G614* and *D614* and detect SARS-CoV-2 and

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D614G mutation. This suggests that SPR can be also used to detect RBD mutations that correlate with the SARS-CoV-2 infectivity.

Furthermore, it is feasible to design specific aptamers as recognition materials for SARS-CoV-2 mutation targets and develop biosensors for detecting other SARS-CoV-2 mutants, including those with mutations in RBD, at the gene level. Various biological cognitive elements and detection principles of biosensor technology can be applied to detect SARS-CoV-2 and its mutation. Common biosensor technology combined with amplification technology,[69] restriction enzyme digestion, CRISPR/Cas technology,[70,71] nanomaterials,[72] and other strategies can be used to identify SARS-CoV-2 mutations. Specific capture and detection probes can be designed to identify the specific mutated sequences to develop an efficient, sensitive, and simple biosensor for mutation detection. The development of biosensors based on electrochemistry, piezoelectricity, fluorescence, surface plasmon resonance, and electrochemiluminescence combined with the signal amplification strategy will be important for the fast and reliable detection of SARS-CoV-2 and its mutation (Figure 2).

Zhao et al. reported the construction of a calixarene-functionalized graphene oxide electrochemical sensor targeting SARS-CoV-2 RNA. Based on the super sandwich recognition strategy, SARS-CoV-2 was detected using portable electrochemical smartphones.[73] Bokelmann et al. tested SARS-CoV-2 instant care batch by combining hybrid capture and improved colorimetric LAMP and analyzed the color based on smartphone APP.[74] Fozouni et al. reported an amplification-free CRISPR-Cas13a method, which was based on a fluorescence microscope and the reaction chamber of mobile phone to quantify Cas13a and analyze the generated fluorescence signal for direct detection of SARS-CoV-2.[75] Smartphone can be applied for the detection at the point-of-care.

Considering the increase of mutations in SARS-CoV-2, it is urgent to detect multiple mutations of SARS-CoV-2 simultaneously with integrating and combining multiple biosensing technologies.[76,77] Multisensor technology can also be used to detect multiple variants of SARS-CoV-2 at the same time, by providing a fast, effective, and reliable point-of-care testing (POCT) for genotyping mutations.

Integrated Biosensors. Integrated biosensors have been applied successfully in the areas of enzyme, DNA, and proteomics analysis, diagnosis, and drug discovery. Biomarker detection in body fluids can lead to disease diagnosis and identification of clinically important physiological parameters. It can also be used for the simultaneous detection of bacteria and viruses.[78] SARS-CoV-2 virus and its mutation can also be detected by multiple integrated biosensors. Microfluidic chip technology allows the manipulation of fluids in microchannels.[79,80] The integration of biosensors and microfluidics has attracted the interest of the scientific community. Most components of biosensors can be miniaturized to form arrays and integrated into the laboratory equipment of chip (lab on a chip). Multiple integrated biosensors improve the analysis ability and provide a miniaturized and low-cost analysis platform.[81] The design and fabrication of novel microfluidic devices require polymers with improved optical, thermal, and mechanical properties. Some materials used in the fabrication of sensor microfluidic devices include paper, glass, silicon, plastic, and polycarbonate.[82] Multiple biosensors integrated in these miniaturized microfluidic devices are widely used to analyze complex samples from lab, environment, and clinical settings. With the advantages in minimal sample preparation and fast operation, multiple analytes in multiple samples can be detected and identified simultaneously.[83]

Integrated biosensors have a series of biochemical recognition elements, which can be transformed into quantifiable optical, mechanical, or electronic signals. Taitt et al. developed a multianalyte array biosensor (MAAB), which can simultaneously detect and identify multiple analytes in complex samples using rapid fluorescence immunoassay. The sensitivity is close to that of gold standard ELISA.[84] Optical sensors based on fluorescence, colorimetry, and surface plasmon resonance have lower detection limits. An optical biosensor does not need any physical contact with the sample, which is compatible with physiological solution, and is insensitive to the change of solution. Compared with the popular optical-based detection, the signal generated by the integrated electrochemical biosensor is electronic, which has the advantages of high sensitivity, small volume, low cost, fast response, easy integration of signal, and compatibility with microprocessing technology. Electrochemical biosensors can be divided into amperometric, voltammetric, impedance, and chrono coulometric biosensors. The shape, size, structural materials, and surface properties of electrodes play an important role in the performance of electrochemical integrated biosensors.[85] Based on the number of tags, multiple electrochemical biosensors can be divided into single tag and multitag integrated biosensors. Crosstalk between microelectrodes is still the main problem in electrochemical array, which should be avoided in the design of integrated biosensors. A field-effect transistor, nanotechnology, and printing technology also bring new opportunities for integrated electrochemical biosensors.[86] Using electrochemiluminescence strategy combined with chip microarray is another integrated biosensor technology.[87]

The ongoing pandemic has shown the urgent need to develop POCT equipment, which can be used for the effective detection of the SARS-CoV-2 and its variants. The integration of microfluidics, arrays, nanomaterials, chips, and biosensors lays the foundation for the design of POCT devices.[88]

MULTIPLEX BIOSENSING TECHNOLOGIES FOR THE DETECTION OF VIRUS

Simultaneous Detection of Various Viruses Based on an Optical Array Biosensor. Optical multiple biosensing technology is a common strategy for multiple virus detection. Jenison et al. designed a thin-film biosensor, on which the capture probe was coated. The nucleic acid hybridization was transformed into a molecular film by the enzyme-catalyzed reaction. The existence of the target sequence changed the interference mode of the biosensor surface, resulting in color change. The technology was applied to chip integration. The capture probe containing the human respiratory virus sequence could simultaneously detect six virus-specific RT-PCR products from infected cell lysates in 10 min. This technique can be widely used in allele identification and detection of infectious diseases.[89] Hu et al. developed a label-free multicolor biosensor combined with exonuclease-assisted autocatalytic target cycle amplification technology for multiple detection of DNA. Exoenzyme III (ExoIII) is a sequence-independent enzyme, which can catalyze the gradual removal of a single nucleotide at the 3′-OH end of double-stranded DNA without specific recognition sites. The biosensor can

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distinguish target DNA simultaneously and polychromatically analyze different oligonucleotides in samples and construct a multichannel DNA detection platform.[90] HBV is a common infectious hepatitis disease. Rapid and effective detection of HBV biomarkers can avoid the risk of infection and prevent the outbreak of HBV.

Biosensors combine fluorescent nanomaterials including quantum dots as signal recognition elements for virus detection. Takemura et al. constructed an immunofluorescence nano biosensor for influenza virus induced by local surface plasmon resonance (LSPR). The QD fluorescence signal induced by AuNPs was combined with antineuraminidase antibody (anti-NAAB) to successfully detect H1N1, H3N2 influenza viruses, and norovirus.[91] Xu et al. developed protein microarray-based biosensors, including single- channel and multichannel arrays, and used SPR technology to detect five biomarkers of HBV serology.[30] Shi et al. reported a biosensor based on SPR. Specific oligonucleotides were fixed on SPR chip, biotin-labeled PCR primers were used, and streptavidin was introduced to amplify the signal after hybridization to detect pharyngeal swab samples of nine common respiratory viruses.[92] The optical chip sensor has different antibody sensing channels as a biological recognition element. The interaction between the target antigen in the sample and their surface-immobilized antibodies leads to the local change of refractive index, so as to detect viruses and other biological hazards.[93] Most SPR biosensor chips are expensive, and it is difficult to reuse them, which limits their commercialization. Yoo et al. developed a reusable magnetic SPR sensor chip, which can repeatedly detect various target molecules in the traditional SPR system. The device was used to detect H1N1 influenza virus with good repeatability and did not need any chemical process to refresh.[94] This method can integrate multichannel chip with its advantages and detect other viruses repeatedly, thus reducing the costs. Antibodies, complementary nucleic acids, and aptamers are the most useful recognition elements for virus recognition.

Antisense oligonucleotide (ASO) is a traditional recognition element, which is a complementary sequence of some unique sites in virus genome. Colloids and nanostructured surfaces have fundamentally different signal amplification mechanisms. They modify aptamers as recognition elements of surface-enhanced Raman spectroscopy (SERS) biosensors for the quantitative and qualitative detection of various viruses.[95] Kim designed an integrated rapid biosensor system for air sampling and simultaneous enrichment of coronavirus and influenza virus. In this study, aerosol viruses including human coronavirus 229E (HCoV-229E), influenza A H1N1 subtype (A/H1N1), and influenza A H3N2 subtype (A/H3N2) were captured by an electrostatic air sampler in continuously flowing liquid and protein-coated magnetic particles for simultaneous detection of hydrosol to hydrosol (HTH)-enriched jet channels. The collected undetectable samples became detectable samples within 10 min during HTH enrichment, and the integrated device was used for virus risk assessment of the field environment.[96] Sciuto et al. described an integrated biosensor platform based on silicon plastic hybrid disk laboratory technology, which can amplify nucleic acid by qRT-PCR and comprehensively extract, purify and detect HBV in an integrated format.[97] Bruls et al. introduced a nanoparticle-based optical magnetic immunoassay technology, which is used for magnetic actuation and optical detection in a fixed sample fluid. Photomagnetic technology can be used to monitor the materials on the binding surface of magnetic nanoparticles with high sensitivity and multichannel integrated detection. This study promoted the development of nursing point diagnosis.[98] Optical array sensor has high precision and strong anti-interference ability.

Simultaneous Detection of Various Viruses Based on an Electrical Array Biosensor. Electrochemical biosensors for virus detection use conductive and semiconductor materials as transducers. The target virus and the electrode immobilized biological recognition element combine with the related chemical energy, which is converted into electrical energy by electrochemical methods involving electrode and electrolyte solution. Based on the electrochemical sensing method, the virus was identified and quantified by immunoassay, DNA-based detection of antibody, and nucleic acid sequence.[99] Biosensors based on semiconductor field-effect devices (BioFEDs) use their intrinsic charge to detect charged biomolecules and biological particles without labels. Different types of biological FEDs have been successfully used to detect a variety of dangerous viruses. The device can detect a number of virus-related parameters (virus particles, virus antigen, virus nucleic acid, and antibody against virus) and provide more accurate and reliable disease diagnosis. The test samples come from a wide range of sources, including whole blood, plasma, serum, urine, saliva, nasopharyngeal swab, or mouthwash. FEDs, plant virus enhancers, and their combinations play an important role in the early diagnosis and treatment of infectious diseases in the future.[100]

Nanotechnology improves the performance of biosensors by changing the properties of materials and particle size.[101] Nanomaterials enhance the affinity, selectivity, and sensitivity of virus detection, for example, carbon nanomaterials, quantum dots, metal nanoclusters, polymer nanocomposites, plasma nanomaterials, nanomaterials, and other nanomaterials. Integrated biosensors based on nanomaterials can be used to detect clinical pathogens of bacteria and viruses.[102] Combining the development of nanomaterials with biosensor technology, a new tool to detect and identify bacteria or viruses can be produced, named nano biosensor. Nano biosensors can improve the sensitivity and specificity of detection, nanoscale integration, and real-time detection, using a variety of label-free sensing mechanisms.[103] Graphene-based biosensors can detect different types of viruses, such as Ebola virus, Zika virus, and influenza. Recent studies have shown that the sensor can detect SARS-CoV-2 virus in clinical samples, SARS-CoV-2 antigen in standard buffer and transport medium, and cultured SARS-CoV-2 virus when SARS-CoV-2 spike antibody binds to graphene sheet in a sensing region.[104] Torrente-Rodríguez et al. mass-produce flexible laser engraving graphene sensor array to detect viral antigen nucleocapsid protein, IgM and IgG antibodies, and inflammatory biomarker C-reactive protein. Hassan et al. reviewed virus detection systems based on plasma metamaterials. Plasma metamaterials have potential for real-time sensing, label-free sensing mechanism, and miniaturization of sensor chips. Based on the fluid characteristics of the sample, the nano plasma biosensor chip was introduced. HIV, coronavirus, influenza, dengue fever, adenovirus, Zika virus, hepatitis, and norovirus have been successfully detected.[105] Two kinds of low pathogenicity common avian influenza viruses (H3N8 and H4N5) were detected in wild ducks, which were well correlated with the results of ELISA. This method has high throughput and requires less serum to obtain more data.[106] Electrochemical biosensors are simple, affordable, and

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compatible with multiple and immediate medical strategies. They are reliable tools for virus detection.

Multiplex Biosensing Technologies for the Detection of SARS-CoV-2 and Its Mutation. Multisensor technology can simultaneously detect multiple viruses, identify SARS-CoV-2, and simultaneously detect multiple biomarkers of SARS-CoV-2. Minjun et al. developed a new one-step quadruple real-time reverse transcription polymerase chain reaction (rRT-PCR) detection method for the diagnosis, differential diagnosis, and coinfection detection of SARS-CoV-2.[31,128] The system was optimized by screening primers and probes. The ORF1ab and N genes of SARS-CoV-2, influenza A virus (hIAV), and influenza B virus (hIBV) were detected and identified simultaneously. Compared with single rRT-PCR, quadruple rRT-PCR can improve the detection efficiency by reducing the number of single detection. It has the advantages of good repeatability and high sensitivity.[129]

The response of specific immunoglobulin G (IgG) and M (IgM) antibody to S, N, and RBD of SARS-CoV-2 occurred within 6−15 days after onset of infection.[130] Johnson et al. reported a multiplex solid-phase chemiluminescence method for simultaneous detection of IgG binding to four SARS-CoV-2 antigens (including SARS-CoV-2 trimer spike, RBD, spike N-terminal domain, and nucleocapsid antigen), quantitative antibody-induced inhibition of ACE2 binding, and evaluation of the function of antispike antibody. This method can be used to detect anti-SARS-CoV-2 antibody within 14 days after the onset of symptoms of COVID-19 with repeatability and high specificity. It can be used to determine the antibody function and has a good correlation with S protein antibody concentration.[131] The performance of the current lateral flow immunoassay device is not enough to meet the requirements of most individual patients with COVID-19. Shaw et al. provided a new and rapid quantitative multiplex gold nanoparticle technology to evaluate the potential of anti-SARS-CoV-2 antigen−antibody detection reliably and accurately, so as to assist clinical diagnosis and decision making. The portable desktop multiplex array technology described in this study has been proved to be effective in detecting antibodies after vaccination. Each array consists of antibodies that capture CRP; proteins A/G that capture total Fc binding antibodies; S1, S2, and N proteins that capture COVID-19 recombinant antigens; SARS membrane (M); and envelope (E) proteins. A quantitative measurement of the response of IgG, IgA, and IgM to SARS-CoV-2 S1, S2, and N proteins is provided.[132] Lin et al. have successfully developed a multi-detection tool for SARS-CoV-2, which integrates a diagnostic chip, self-made portable fluorescence detector, and microfluidic immunoassay technology. The chip consists of a sample loading chamber, a waste container, and a fluid channel for fluorescence immunoassay including a capture area and a test area. The fluorescence detection analyzer has the functions of centrifugation, fluorescence detection, and result display. Microfluidic immunoassay is used for sensitive and simultaneous detection of IgG/IgM/antigen of SARS-CoV-2 within 15 min. A portable microfluidic immunoassay system has been established, which is convenient for high-throughput, sensitive, and rapid detection (<15 min). At the same time, the IgG/IgM/antigen of SARS-CoV-2 can be detected many times and on the spot. The system is expected to be used for quantitative and sensitive detection of biomarkers of various diseases.[133]

Microarray studies detect point mutations and SNPs in multiple samples in parallel.[134] Multiplicity allows the quantification of multiple analytes in one step, providing advantages over a single test by shorter processing time, lower sample volume, and lower cost per test. SARS-CoV-2 can mutate in vivo and in the process of transmission and cross infect in the process of human and environmental transmission. SARS-CoV-2 has been spread in many common ways, and individual mutant strains have become the mainstream, whether it was *D614G*, *N501Y*, and *P681H* mutation, or *69/70 deletion* in B.1.1.7(Alpha) lineage, or *K417T*, *E484K*, and *N501Y* mutation in both B.1.351 and P.1 lineages. *Y501* in mutant RBD can coordinate well with *Y41* and *K353* in ACE2 through hydrophobic interaction, which improves the binding affinity of RBD with ACE2.[28,33] Compared with wild-type strains, both of them spread rapidly in the population and may cause more serious diseases. Both vaccination and natural infection against SARS-CoV-2 can produce a "polyclonal" response to the S protein. The accumulation of multiple mutations in S protein may evade the ability of natural immunity or vaccine-induced immunity, but the impact of the known mutations and those which might emerge in the future on the existing vaccines will require further investigation. Most commercial PCR tests have multiple targets for virus detection, so even if the mutation affects one of the targets, other PCR targets will still work.[135]

SARS-CoV-2 mutations can be detected at the protein level and gene level. Biosensor technology is also widely used in the detection of virus mutation.[136] The mutation of ACE2 binding S protein plays a key role. We can design a biosensor to detect SARS-CoV-2 mutant protein by ACE2 protein. Biosensors based on aptamers and antibodies can be designed to distinguish mutated S protein. Multiple mutations of SARS-CoV-2 can be detected simultaneously using multiple biosensor technology. Antibodies or aptamers that specifically recognize S protein, N protein, and RBD mutation of SARS-CoV-2 can be integrated into a chip for simultaneous detection as biological recognition elements. The application of biosensor technology based on the gene level is more promising. RT-PCR and gene sequencing were used for detection, combined with different signal amplification techniques. It was applied to the electrochemical and photochemical detection of SARS-CoV-2.[69,70] The objective was to design and optimize the primers in accordance with SARS-CoV-2 mutation to realize the detection of SARS-CoV-2. Sequencing technology provides high throughput and accurate genotyping of SARS-CoV-2.[137] Multiple mutations of SARS-CoV-2 were detected by electrochemical and optical techniques, lateral flow strip, sequencing, or microarray. The hybridization technique using point mutation probe and corresponding perfect matching probe can also be used for SARS-CoV-2 mutation detection reliably. Microarray hybridization can be used to identify single base mismatches from small samples to thousands of samples, using dozens to hundreds of probes. Microarray technology can be used for rapid and accurate point mutation detection and early clinical diagnosis of SARS-CoV-2 mutation.[138]

Future Development of Multiplex Integrated Biosensors. At present, the most common SARS-CoV-2 nucleic acid detection uses nasopharyngeal swabs. The biosensor array for detection of SARS-CoV-2 and its mutation can be integrated into a small platform for increased portability. The results of biosensor detection can be interpreted, and the automatic quantitative detection of SARS-CoV-2 and mutation can be performed with the help of a smartphone APP. Novel

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portable biosensors can be used for real-time detection and remote monitoring of patients with COVID-19.[139] A number of novel biosensors for the reliable and efficient diagnosis of pathogens have been designed recently. The paper-based microfluidic bioassay does not require complex equipment, reagents, or power supply. It is low-cost, fast, sensitive, accurate, and easy to use and transport. A paper biosensor can also be applied for the detection of SARS-CoV-2.[140]

In the future, digital biosensor technology can be used to develop digital bar code particles and a microsimulation architecture for multiplex analyte quantification. Real-time reading through a mobile phone is important for POCT diagnosis. It is dedicated to the functionalization of physical bar code particles and has specific antibodies that are crucial to the attachment of biomarkers on the cell surface. It can be used to detect SARS-CoV-2 biomarkers.[141]

Clustering regularly spaced short palindromic repeats (CRISPR) is a technology that won the Nobel Prize in chemistry in 2020. CRISPR is a special genomic DNA sequence derived from an acquired immune system in bacteria and archaea. It can recognize and destroy the invading virus by a special enzyme. The purified Cas9 protein is a double RNA-directed endonuclease. It has been shown that the CRISPR-Cas9 system can cut any DNA strand and modify genes in living cells. Consequently, CRISPR has become the most popular gene-editing technology. CRISPR/CAS is an RNA-directed adaptive immune system, including programmable CAS endonuclease and CRISPR RNA (crRNAs), which can be used for nucleic acid recognition and editing.[73] In recent years, the nucleic acid detection technology based on the CRISPR/CAS system has developed rapidly, mainly relying on the technical principle that the system can combine CRISPR-associated enzymes (including Cas12, Cas13, Cas14) with the target sequence and cleave it under the guidance of single-stranded guide RNA.[74] The specific high-sensitivity enzyme report unlock (SHERLOCK) technology developed combines various CRISPR/CAS enzymes. Based on nucleic acid isothermal amplification combined with the CRISPR/CAS nucleic acid detection system, it can simultaneously detect a variety of nucleic acids, which is suitable for rapid diagnosis including infectious diseases and sensitive genotyping.[142] Biosensor technology based on CRISPR/CAS has been applied to detect hepatitis virus, influenza virus, and others. Recent studies have shown that the Sherlock technology based on CRISPR can detect SARS-CoV-2, and the synthetic RNA fragment of the SARS-CoV-2 virus can detect the AM level internal target sequence within 1 h.[75] However, there is still a gap in the research on the construction of a CRISPR/CAS sensor array for different SARS-CoV-2 mutations.

CRISPR protein is a nuclease, which can decompose viral nucleic acid. It can be used for sensitive, cheap, and rapid detection of viruses and bacteria. The CRISPR/CAS system has been successfully used to detect virus mutations. SARS-CoV-2 mutants have several potential CAS crRNA targets. The binding of Cas13 to the SARS-CoV-2 mutant gene activates trans cleavage Cas13, which cleaves the specific RNA strands and activates the CRISPR/Cas system, thus producing a bright signal. A SARS-CoV-2 mutation array sensing platform based on the CRISPR/CAS system can be constructed. Finally, the mobile phone intelligent detection software can be designed to read the signal characteristics of the test strip for automatic detection and analysis. Reliable and efficient detection of multiple SARS-CoV-2 mutations will require optimizations of the conditions and crRNA screening.

Biosensor manufacturing using nano/micromanufacturing methods, material chemistry, electronics, bioelectronics, and other fields of innovative technology and scientific progress, coupled with digital communication technology and wireless sensor network integration, is among the main development trends in this exciting area of research.

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Notes

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